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Abstract: OBJECTIVE The aim of this study was to confirm previous observations that proenkephalin A (PENK-A) may serve as prognostic marker in the setting of acute ischemic stroke in a large stroke cohort. METHODS The plasma concentration of PENK-A was measured within 72 hours of symptom onset in 320 consecutively enrolled patients with stroke. The primary outcome measures were unfavorable functional outcome (modified Rankin Scale score 0-2 vs 3-6) and mortality within 90 days. Logistic and cox proportional regression analyses were fitted to estimate odds ratios (ORs), hazard ratios (HRs) and 95% confidence intervals (CIs), respectively, for the association between PENK-A and the primary outcome measures. RESULTS After adjusting for demographic and vascular risk factors, PENK-A was neither independently associated with functional outcome (OR: 1.29, 95% CI: 0.16-10.35) nor mortality (HR: 1.02, 95% CI: 0.14-7.33). CONCLUSION Among patients with acute stroke, PENK-A does not serve as an independent prognostic marker in this external validation cohort.

DOI: https://doi.org/10.1177/1076029619895318

Clinical and Applied Thrombosis/Hemostasis
Volume 26: 1-7

DOI: 10.1177/1076029619895318
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Proenkephalin A Adds No Incremental Prognostic Value After Acute Ischemic Stroke

Philipp Gruber, MD, MSc[1,2], Felix Fluri, MD[3,4], Juliane Schweizer, MD[2], Andreas Luft, MD[2], Beat Müller, MD[5], Mirjam Christ-Crain, MD[6], and Mira Katan, MD, MSc[2]

Abstract

Objective: The aim of this study was to confirm previous observations that proenkephalin A (PENK-A) may serve as prognostic marker in the setting of acute ischemic stroke in a large stroke cohort. **Methods:** The plasma concentration of PENK-A was measured within 72 hours of symptom onset in 320 consecutively enrolled patients with stroke. The primary outcome measures were unfavorable functional outcome (modified Rankin Scale score 0-2 vs 3-6) and mortality within 90 days. Logistic and cox proportional regression analyses were fitted to estimate odds ratios (ORs), hazard ratios (HRs) and 95% confidence intervals (CIs), respectively, for the association between PENK-A and the primary outcome measures. **Results:** After adjusting for demographic and vascular risk factors, PENK-A was neither independently associated with functional outcome (OR: 1.29, 95% CI: 0.16-10.35) nor mortality (HR: 1.02, 95% CI: 0.14-7.33). **Conclusion:** Among patients with acute stroke, PENK-A does not serve as an independent prognostic marker in this external validation cohort.

Keywords

proenkephalin A, blood biomarker, ischemic stroke, prognosis

Date received: 7 August 2019; revised: 6 November 2019; accepted: 19 November 2019.

Introduction

Ischemic stroke is still one of the most challenging health-care burdens globally.[1,2] Stroke prognosis is dependent on stroke severity, infarct location, stroke etiology, age, and comorbidities. Accurate prediction is difficult, and incremental prognostic information is needed to optimize risk stratification. A deeper knowledge of novel factors associated with stroke outcome is desirable. Accurately measured, externally validated prognostic biomarkers are sparse.[3] Several blood biomarkers have been assessed, but only few have been validated in independent studies. Among the most prominent are copeptin and natriuretic peptides with an incremental prognostic value,[4,5] but none of those has found its way into clinical routine.[3]

Proenkephalin A (PENK-A) is a stable precursor protein fragment of the enkephalin (ENK) neuropeptide family.[6] Enkephalin is involved in different biological pathways.[7] In experimental models, ENK reduces cerebral edema after ischemic stroke pointing to a neuroprotective role.[8] Proenkephalin A has been shown to be a potent marker for blood–brain barrier integrity.[9] A product of the precursor peptide PENK-A is elevated in patients with acute stroke and was associated with poor outcome in intracranial hemorrhage (n = 202) or subarachnoid hemorrhage (SAH; n = 360).[10-12] Another study (n = 189) suggested that PENK-A plasma levels might serve as a good prognostic marker in patients with acute stroke for unfavorable outcome.[13] Thus, a higher PENK-A plasma level might be associated with poor clinical outcome in patients with acute stroke.

[1] Department of Neuroradiology, Cantonal Hospital Aarau, Switzerland
[2] Department of Neurology, University Hospital of Zurich, Switzerland
[3] Department of Neurology, University Hospital of Würzburg, Germany
[4] Department of Neurology, Cantonal Hospital of St Gallen, Switzerland
[5] Medical University Clinic, Cantonal Hospital Aarau, Switzerland
[6] Department of Endocrinology, University Hospital of Basel, Switzerland

Corresponding Author:
Philipp Gruber, Departments of Neuroradiology, Cantonal Hospital Aarau, Tellstrasse 25, 5001 Aarau, Switzerland; Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, 8091 Zurich, Switzerland.
Email: philipp.gruber@ksa.ch

The aim of this study was to validate previous observations of the predictive role of PENK-A in a larger cohort of patients with stroke designed for the evaluation of prognostic blood biomarkers.

Methods

Study Setting

The design of the prospective cohort used in the present study has been described in detail elsewhere.[14] Initially, 605 patients with suspected ischemic stroke presenting at the emergency department of the University Hospital Basel in Switzerland were screened. Ischemic stroke was defined according to the World Health Organization criteria as an acute focal neurological deficit lasting longer than 24 hours with no sign of acute intracranial bleeding on cerebral imaging.[15] Thus, the only exclusion criteria for this study was evidence of intracranial bleeding on initial imaging.[14]

Of these 605, 362 patients had an ischemic stroke, 359 completed follow-up (99.2%); and in 320 (89%) patients, ethylenediaminetetraacetic acid (EDTA) plasma was available for PENK-A measurement.

Standard Protocol Approvals, Registrations, and Patients Consent

This study was approved by the local ethics committee and was conducted according to the principles expressed in the Declaration of Helsinki. From all patients, written informed consent was obtained. This study reports on a trial of stroke blood biomarkers (ClinicalTrials.gov: NCT00390962).

Clinical Baseline Variables and Diagnostic Work-Up

The following data were collected with a standardized bed-side interview and complete chart review on admission: vital signs, comorbidities as assessed by the Charlson Comorbidity Index adjusted for stroke,[16] medication prior to ischemic stroke, and cardiovascular risk factors (ie, age, gender, smoking habits, history of hypercholesterolemia, hypertension, diabetes mellitus, previous stroke, positive family history for myocardial infarction, stroke, and history of coronary heart disease). Stroke physicians prospectively recorded the National Institutes of Health Stroke Scale score (NIHSS) upon admission.[17] The patients were clinically classified into 1 of the 4 subtypes of the Oxfordshire Community Stroke Project classification, which differentiate between lacunar stroke, partial and total anterior circulation stroke (TACS), as well as posterior circulation stroke (POCS).[18] Stroke etiology was determined according to the criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification,[19] which distinguishes large-artery atherosclerosis, cardioembolism, small-artery occlusion, other etiology, and undetermined etiology. Computed tomography was performed in all patients on admission to exclude intracranial hemorrhage. Additionally, magnetic resonance imaging with diffusion-weighted imaging was performed in 178 (56%) patients. The lesion size was categorized into 3 different subtypes according to the volume in small lesions (<10 mm^3), medium lesions (10-100 mm^3), and large lesions (>100 mm^3).[20]

Blood Sampling

In all patients, routine blood analyses were performed directly upon admission including C-reactive protein (CRP), creatinine, glucose, uric acid, cholesterol, triglycerides, and white blood cells. In addition, serum and EDTA tubes were collected on admission within 72 hours from symptom onset. These materials were directly centrifuged, aliquoted, and frozen at −80°C for future analyses.

Measurement of PENK-A. Plasma levels of PENK-A were measured in a batch blinded to clinical outcomes. In brief, a chemoluminescence sandwich immunoassay detected midregional PENK A 119-159 with monoclonal antibodies against aminoacid sequence 121 to 134 as described previously (BRAHMS GmbH, Henningsdorf, Germany).[9] The intra-assay and inter-assay coefficients of variations were <10% and <15%, respectively. Median levels in healthy controls are 62.3 pmol/L (range 41.8-131 pmol/L).[9]

Outcome Measures

Primary outcome measures were unfavorable functional outcome defined as a modified Rankin Scale (mRS) score of >2 points and mortality of any cause. Trained stroke physicians or study nurses assessed clinical outcome with a structured follow-up telephone interview, 3 months after the acute stroke. Interviewers were blinded to PENK-A levels and baseline clinical variables.

Statistical Analysis

Discrete variables are expressed as frequency (percentage) and the not normally distributed variables as medians with interquartile ranges (IQR, 25th-75th percentiles). The distribution of raw biomarker data was skewed. After log transformation with a base of 10, the distribution of the biomarker data approximated a normal distribution by plotting the data and considering them visually. Analyses were done for both primary outcome measures separately. Two group comparisons for categorical baseline measurements were performed by chi-square test and for continuous, not normally distributed baseline data by the Wilcoxon rank-sum test. Linear regression analysis was performed for all interval-scaled variables.

To assess the independent association of PENK-A level with functional outcome, logistic models with odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. For mortality, Cox regression models with hazard ratio (HR) were used. Multivariate models were calculated to adjust for possible confounding factors. These final multivariate models included variables significantly associated with an unfavorable outcome

or mortality in the univariate analyses (ie, age, NIHSS on admission, Charlson comorbidity index, heart failure, atrial fibrillation, small artery disease, TACS, and POCS). A P value $< .05$ was considered statistically significant. Finally, we conducted univariate interaction analysis with the baseline characteristics. The statistical analysis was performed with STATA 14.2 (StataCorp LLP, Texas).

Results

Study Population

The distribution of demographic and vascular risk factors was not significantly different between the original cohort of 359 patients and the 320 patients with available blood samples.

The median age of the analyzed cohort of 320 patients was 75 years (IQR 65-82, in the original cohort 75 years [IQR 63-83]), 41% (original cohort 41%) of the patients were women. The most common cardiovascular risk factor was arterial hypertension (76% of patients [original cohort 77%]). The median NIHSS score on admission was 5 (IQR 2-10; original cohort 5 [IQR 2-10]) and the median Charlson comorbidity Index was 1 (IQR 0-2; Table 1). In our cohort, 20% of the patients received an acute stroke treatment (intravenous thrombolysis or endovascular treatment).

Association of PENK-A Plasma Level With Baseline Demographic and Vascular Risk Factors

Median PENK-A plasma level in women (158.5 pg/mL, IQR: 13.25-199.5) was significantly higher compared to median PENK-A plasma level in men (139.0 pg/mL, IQR: 120-168; $P = .0011$). Plasma levels of PENK-A increased slightly with age ($r = 0.27$; $P < .000$) and were considerably higher in octogenarians (165.75 pg/mL, IQR 131.0-206.0) compared to nonoctogenarians (139.0 pg/mL, IQR: 120.0-165.5), $P < .001$. In addition, there was a positive correlation between PENK-A plasma levels and creatinine plasma levels ($r = 0.5$; $P = .000$), as well as between PENK-A plasma level and uric acid ($r = 0.31$, $P = .000$), but not with other laboratory parameters such as CRP and glucose. We found a negative and very weak correlation with body weight ($r = -0.01$; $P = .048$).

Median PENK-A plasma levels were higher in patients with heart failure (184 pg/mL, IQR: 133.5-213.0) compared to patients without heart failure (145.5 pg/mL, IQR: 120-171.75; $P = .0004$). The same was true for patients with atrial fibrillation (165.5 pg/mL, IQR: 135.5-203.5) compared to patients without atrial fibrillation (143.5 pg/mL, IQR 120-174; $P = .0007$), as well as patients with coronary heart disease (161 pg/mL, IQR: 132.5-203.5) compared to patients without coronary heart disease (144.5 pg/mL, IQR: 119-172.5; $P = .0018$).

There was neither a correlation between PENK-A plasma levels and the NIHSS score on admission nor a correlation between PENK-A plasma levels and diffusion weighted imaging lesion size, both surrogate marker for stroke severity. Finally, there was also no association with any of the TOAST subgroups (Table 1).

Table 1. Baseline Characteristics of All Patients.

	All Patients Included in This Analysis (N = 320)	Original Cohort (N = 359)
Demographic data		
Women, no. (%)	131 (41%)	149 (41%)
Age (median-IQR)	75 (62-82)	75 (63-83)
Medical history, n (%)		
Hypertension, no. (%)	243 (76%)	275 (77%)
Arterial fibrillation, no. (%)	55 (17%)	75 (21%)
Coronary heart disease, no. (%)	82 (26%)	91 (25%)
Heart failure, no. (%)	44 (14%)	
Dyslipidemia, no. (%)	81 (25%)	93 (26%)
Diabetes mellitus, no. (%)	65 (20%)	71 (20%)
Current smoker, no. (%)	111 (35%)	124 (35%)
Previous cerebrovascular event, no. (%)	79 (25%)	88 (25%)
Charlson comorbidity index, no. (%)	1 (0-2)	1 (0-2)
Clinical data		
NIHSS at admission (median-IQR)	5 (2-10)	5 (2-10)
MR imaging data[a]		
DWI lesion size (mm^3, median-IQR)	1.6 (0.2-21)	
OCSP, n (%)		
TACS	36 (11%)	41 (11%)
PACS	139 (44%)	162 (45%)
LACS	69 (22%)	74 (21%)
POCS	75 (23%)	83 (23%)
Laboratory values, median (IQR)		
PENK-A (pg/mL)	147.75 (123.5-183.25)	
CRP (mg/L)	3.5 (3-9.7)	3.6 (3.0-9.9)
Creatinine (mmol/L)	75 (63-88)	
TOAST subtype, n (%)		
Large-vessel disease	62 (19%)	65 (18%)
Cardioembolic	109 (34%)	131 (36%)
Small-artery disease	50 (16%)	55 (15%)
Other	16 (5%)	16 (4%)
Undetermined	83 (26%)	92 (26%)

Abbreviations: CRP, C-reactive protein; DWI, diffusion weighted imaging; IQR, interquartile range; LACS, Lacunar anterior circulation stroke; MR, magnetic resonance; NIHSS, National Institute of Health Stroke Scale; OCPS, Oxfordshire Community Stroke Project score; PACS, partial anterior circulation stroke; POCS, posterior circulation stroke; TACS, total anterior circulation stroke; TOAST, Trial of Org 10172 in Acute Stroke Treatment Classification.
[a]DWI lesion size only for n = 179 available.

Association of PENK-A With 90-Day Functional Outcome and Mortality

Patient with an unfavorable outcome revealed significantly higher median PENK-A plasma level (160.75 pg/mL, IQR:

Table 2. Significant Univariate and Multivariate Logistic Regression Analysis for Functional Outcome.[a,b]

Logistic Regression Model	Univariate Analyses			Multivariate Analyses		
Predictors	OR	95% CI	*P*	OR	95% CI	*P*
Log PENK-A (pg/mL)[c]	10.67	2.31-49.38	**.002**	1.95	0.27-13.72	.50
Log CRP (mg/L)[c]	3.00	1.72-5.25	**<.001**			
Age (years)	1.06	1.04-1.09	**<.001**	1.07	1.04-1.10	**<.001**
NIHSS score	1.16	1.11-1.21	**<.001**	1.16	1.10-1.23	**<.001**
Charlson index score	1.38	1.17 - 1.61	**<.001**	1.39	1.14-1.72	**.001**
Atrial fibrillation	2.07	1.15-3.73	**.015**	0.57	0.25-1.31	.19
DWI lesion size (mm^3)[d]	2.51	1.49-4.24	**.001**			
Heart failure	2.56	1.15-3.73	**.005**	1.30	0.56-3.04	.54
Small-artery disease	0.44	0.22-0.86	**.017**	0.87	0.37-2.04	.75
Undetermined	1.7	1.02-2.82	**.039**	1.20	0.60-2.41	.60
TACS	0.51	0.29-0.9	**.02**	2.22	0.77-6.41	.14
POCS	0.6	0.47-0.77	**<.001**	0.84	0.41-1.72	.63

Abbreviations: CI, confidence interval; CRP, C-reactive protein; DWI, diffusion weighted imaging; HR, hazard ratio; NIHSS, National Institute of Health Stroke Scale; OR, odds ratio; PENK-A, proenkephalin A; POCS, posterior circulation stroke; TACS, total anterior circulation stroke.
[a]Because date of CRP and DWI is only available in a subgroup of patients, these parameters were not included in the final multivariate analysis.
[b]CRP (n = 262).
[c]Log10-transformed values were used in these analyses.
[d]DWI, diffusion weighted imaging (n = 179).
Note: The boldface values will highlight that this values are statistically significant values.

127.0-194.5) compared to patients with favorable outcome (142.5 pg/mL, IQR: 120.0-166.0), $P = .0033$.

In the univariate logistic regression analysis, patients with a high PENK-A plasma level (OR 10.67, 95% CI: 2.31-49.38) were more likely to have an unfavorable outcome (Table 2). But, after adjusting for all other significant predictors of the univariate analysis, PENK-A levels were no longer associated with functional outcome (Table 2). Only age (OR 1.07, 95% CI: 1.04-1.10), NIHSS score on admission (OR 1.16, 95% CI: 1.10-1.23), and Charlson comorbidity index score (OR 1.39, 95% CI: 1.14-1.72) remained independently associated with functional outcome after adjustment (Table 2).

Similar results are found for mortality as the other primary outcome measures. In this cohort, a total of 39 patients died within 90 days after the stroke event. Median PENK-A plasma level of these patients (160.0 pg/mL, IQR: 127.0-202.0) was higher compared to the median PENK-A plasma level of the survivors but not statistically significant (146 pg/mL, IQR: 123.0-181.5; $P = .069$). The univariate cox regression model for mortality showed a significant association for high PENK-A plasma level with mortality (HR 8.61, 95% CI: 1.61-45.7; Table 3). In the multivariate analysis, only age (HR 1.07, 95% CI: 1.03-1.11) and NIHSS score on admission (HR 1.11, 95% CI: 1.07-1.16) remained independently associated with mortality within 90 days after stroke.

We found no interaction of PENK-A levels regarding functional outcome with age (p for interaction [pi] = 0.81), gender (pi = 0.78), heart failure (pi = 0.24), atrial fibrillation (pi = 0.63), renal insufficiency (pi = 0.13), or acute stroke treatment

Table 3. Significant Univariate and Multivariate Cox Regression Model for Mortality.

Cox Regression Model	Univariate Analyses			Multivariate Analyses		
Predictors	HR	95% CI	*P*	HR	95% CI	*P*
Log PENK-A (pg/mL)[a,b,c]	8.61	1.61-45.7	**.012**	1.02	0.14-7.33	.98
Log CRP (mg/L)[a,b,c]	2.85	1.62-5.02	**<.001**			
Age (years)	1.08	1.05-1.13	**<.001**	1.07	1.03-1.11	**<.01**
NIHSS score	1.13	1.1-1.16	**<.001**	1.11	1.07-1.15	**<.01**
Charlson index score	1.16	1.98-1.37	**.091**			
Atrial fibrillation	3.38	1.77-6.44	**<.001**	0.67	0.31-1.45	.31
DWI lesion size (mm^3)[d]	3.45	1.55-7.66	**.002**			
Heart failure	2.38	1.16-4.89	**.018**	2.97	1.33-6.64	.08
Small-artery disease	0.13	0.02-0.97	**.047**	0.28	0.04-2.12	.22
Undetermined	1.89	0.99-3.6	.052			
TACS	5.44	2.83-10.47	**<.001**	2.09	0.98-4.49	.057
POCS	0.36	0.13-0.99	**.05**	0.73	0.24-2.18	.57

Abbreviations: CI, confidence interval; CRP, C-reactive protein; DWI, diffusion weighted imaging; HR, hazard ratio; NIHSS, National Institute of Health Stroke Scale; PENK-A, proenkephalin A; POCS, posterior circulation stroke; TACS, total anterior circulation stroke; OR, odds ratio.
[a]Because of numerical imbalance CRP and DWI were not included in the final multivariate analysis.
[b]CRP (n = 262).
[c]Log10-transformed values were used in these analyses.
[d]DWI (n = 179).
Note: The boldface values will highlight that this values are statistically significant values.

(pi = 0.59); or PENK-A levels regarding mortality with age (pi = 0.55); gender (pi = 0.06), heart failure (pi = 0.14), atrial fibrillation (pi = 0.83), renal insufficiency (pi = 0.44) or acute stroke treatment (pi = 0.11).

Discussion

This study of 320 patients with acute stroke could not confirm and validate that PENK-A plasma levels were independently associated with functional outcome or mortality within 90 days after index stroke. We found no incremental value of PENK-A as prognostic marker in the acute stroke setting. The initial association of PENK-A levels with functional outcome and mortality was attenuated and most likely confounded by other comorbidities.

So far, there has been one promising study looking at PENK-A levels and stroke outcome, in which high PENK-A plasma levels were significantly and independently associated with an unfavorable functional outcome and mortality in patients with acute stroke.[13]

In addition, there is evidence from studies in patients with other cerebrovascular diseases such as spontaneous intra-cerebral hemorrhage or SAH, which showed that high PENK-A plasma levels were independently associated with unfavorable outcome.[10-12] In contrast, in the unadjusted analyses, we only found an association of PENK-A levels with an unfavorable outcome (mRS > 2), suggesting that the association was confounded by demographic and other vascular risk factors. Compared to the above-mentioned smaller study (n = 124),[13] the current study analyzed almost 2.5 times as many patients (n = 320). Due to potentially insufficient power, the positive result in other studies may not reflect a real-independent pathophysiological impact of PENK-A in acute stroke. In addition, we used more variables for adjustment in our model such as comorbidities reflected by the Charlson comorbidity index, stroke etiology, and stroke syndrome.

Additionally, we could not confirm a direct correlation of stroke lesion volume and PENK-A levels as has been shown by others.[13] This challenges the assumption that PENK-A levels are related to blood–brain barrier leakage.[9,13]

Presumably, this contradictory finding may be partially explained by the fact that ENKs are not only specifically expressed in central neuronal tissue but also expressed in the peripheral neuronal tissue of gastrointestinal tract or the lung, the heart, the skeletal muscle, the kidney, and immune cells.[21-23] Furthermore, the biological role of this neuropeptide family is not fully understood; they are involved in many different pathways such as immune stimulation, nociception, or stress response,[9] thus not specific to ischemic tissue damage during acute stroke.

Our results demonstrate a close association between PENK-A level and cardiac comorbidities. Higher PENK-A plasma level was found in patients with heart failure, atrial fibrillation, or coronary heart diseases. These findings are consistent with previous studies reporting that high PENK-A levels predict a poor outcome in patients with acute myocardial infarction. In addition, high PENK-A plasma level may be also associated with unfavorable course of heart failure.[24,25] This may be related to the fact that cardiomyocytes produce ENKs which influence blood pressure and heart rate.[26] Since blood pressure and cardiac activity are usually increased in acute ischemic stroke, higher PENK-A plasma level may be partially explained by this cardiac ENK effect. But, there was no significant association to the cardioembolic subtype according to the TOAST classification. Thus, PENK-A is not suitable to discriminate stroke subtypes. We hypothesize that the missing association of PENK-A levels with specifically cardioembolic stroke etiology despite the link with underlying cardiac comorbidities is due to the fact that probably other factors such as renal insufficiency influence PENK-A levels even more pronounced. Moreover, we found that age is a strong confounder but revealed no interaction.

In our multivariate analyses, NIHSS, age, as well as the Charlson comorbidity index for functional outcome remained the most relevant independent prognostic factors for functional outcome and mortality in patients with acute stroke. This is inline with several outcome prediction studies as well as validated clinical prognostic scores, suggesting that our cohort seems to be representative of patients with ischemic stroke.[27-30]

Despite our relatively large sample size and representative stroke population, some limitations merit attention. First, PENK-A was not a prespecified marker in this cohort study but analyzed post hoc. Second due to single measurement within 72 hours (with 89% within 24 hours) from stroke onset, the current analyses provide no information on the kinetics of PENK-A after stroke. Therefore, we cannot draw any conclusions about the prognostic role beyond 72 hours of stroke onset. However, it is debatable if prognostic information for risk stratification after 72 hours of stroke onset is clinically relevant.

Conclusion

In this study, PENK-A failed to be an independent prognostic biomarker for unfavorable outcome or mortality among patients with acute ischemic stroke. This result may be related to the fact that PENK-A is not organospecific and is involved in different pathways and may be strongly influenced by other known vascular risk factors, thus not able to add incremental value for stroke risk stratification beyond the NIHSS and age. This study underlines the relevance of external validation, as blood biomarkers typically report higher effect size in pilot studies compared to subsequent larger validation studies of the same biomarker.[27]

Authors' Note

Ethical approval to report this study was obtained from the local Ethic committee board of University Hospital Basel (EKBB 157/06). This study reports on a trial of stroke blood biomarkers (ClinicalTrials.gov: NCT00390962). Written informed consent was obtained from the

patient(s) for their anonymized information to be published in this article.

Acknowledgments

The PENK-A chemoluminescence assay was offered to MK and MCC from BRAHMS GmbH, Hennigsdorf, Germany.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was founded by the Swiss National Science Foundation (PZ00P3_142422 [MK]).

ORCID iD

Philipp Gruber https://orcid.org/0000-0001-8464-3051

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